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# Application of Multi-Component Damage Assessment Model (MDAM) for the Toxicity of Metabolized PAH in *Hyalella azteca*

JONG-HYEON LEE† AND PETER F. LANDRUM*,‡

*Cooperative Institute for Limnology and Ecosystems Research, University of Michigan, Ann Arbor, Michigan 48105-1593, and Great Lakes Environmental Research Laboratory, National Oceanic and Atmospheric Administration, Ann Arbor, Michigan 48105-1593*

Biotransformation and time-dependent toxicity of pyrene and fluorene, in the presence and absence of the biotransformation inhibitor 6-propylpiperonyl butyl diethylene glycol ether known as piperonyl butoxide (PBO), were investigated in *Hyalella azteca*. Toxicokinetics and biotransformation were determined in both short- and long-term experiments. For pyrene, the uptake rate coefficient ($k_u = 99 \pm 9$ L kg$^{-1}$ h$^{-1}$), elimination rate constant for parent compound ($k_{ep} = 0.050 \pm 0.008$ h$^{-1}$), biotransformation rate constant ($k_m = 0.016 \pm 0.003$ h$^{-1}$), and elimination rate constant for metabolites ($k_{em} = 0.021 \pm 0.007$ h$^{-1}$) were determined from a short-term study. For fluorene, $k_u$ ($130 \pm 30$ L kg$^{-1}$ h$^{-1}$) and $k_{ep}$ ($1.7 \pm 0.2$ h$^{-1}$) were estimated based on $k_m$ ($0.015 \pm 0.002$ h$^{-1}$) and $k_{em}$ ($0.011 \pm 0.002$ h$^{-1}$) determined from a long-term study. At steady state, the percent metabolites for pyrene and fluorene were 43% and 58%, respectively, determined from $k_m/(k_m + k_{em})$. Time-dependent toxicity was determined as both lethal water concentration and lethal body residue in the presence and absence of PBO. Co-exposure to PBO decreased the median lethal residue ($LBR_{50}$) for parent pyrene and shortened the median lethal time ($LT_{50}$). Pyrene toxicity was explained by the body residues of parent pyrene and PBO, where the metabolites' contribution was negligible. For fluorene, co-exposure to PBO increased the $LBR_{50}$ for parent fluorene and shortened the $LT_{50}$. Thus, fluorene metabolites contributed significantly to the toxicity. Using a multicomponent damage assessment model, the toxicodynamic parameters, damage accrual rate coefficient, and damage recovery rate constant for parent pyrene and parent fluorene were very similar and estimated to be from 0.009 to 0.020 $\mu$mol$^{-1}$ g h$^{-1}$ and from 0.003 to 0.013 h$^{-1}$, respectively, and the incipient $LBR_{50}$ at the infinite time ($LBR_{50}(t = \infty)$) was from 0.24 to 0.46 $\mu$mol g$^{-1}$, respectively. These values are similar to the reported $LBR_{50}(t = \infty)$ for other nonpolar narcotics such as pentachlorobenzene (PCBz, $0.38 \pm 0.13$ $\mu$mol g$^{-1}$) and dichlorophenylchloroethylene (DDE, $0.41 \pm 0.19$ $\mu$mol g$^{-1}$). For fluorene metabolites, these values were $0.10 \pm 0.03$ $\mu$mol$^{-1}$ g h$^{-1}$, $0.034 \pm 0.021$ h$^{-1}$, and $0.33 \pm 0.23$ $\mu$mol g$^{-1}$, respectively. However, for pyrene metabolites, since toxicity of pyrene metabolites was negligible, the parameters could not be estimated.

* Corresponding author phone: 734-741-2276; fax: 734-741-2055; e-mail: peter.landrum@noaa.gov.
† University of Michigan.
‡ National Oceanic and Atmospheric Administration.

## Introduction

Polycyclic aromatic hydrocarbons (PAH) are known to be metabolized by several invertebrate species (*1*−*4*). Biotransformation reduces the bioconcentration potential of the parent PAH by increasing the total elimination process, and produces metabolites, which may or may not be readily eliminated (*5*−*7*). In the case of benzo[*a*]pyrene, the body residue of the parent compound could be less than 10% of the total body residue, parent compound plus metabolites, in a species with active biotransformation such as a marine polychaete (*4*).

Some researchers have assumed that the mode of toxic action of the parent PAH and phase I PAH metabolites are similar (*8*) and the toxicities are quantitatively equal, leading to a total body residue as a dose metric related to toxic effect (*4*). However, PAH biotransformation may result in the formation of metabolites that are either detoxified (*6*, *9*) or activated compared to the parent compound (*10*−*11*). Thus, the toxic effect of metabolized organic compounds must be assessed as a mixture of the parent compound and metabolites with different toxicokinetics and toxicodynamics.

The toxicokinetics of PAH including biotransformation in aquatic invertebrates has been studied in only a few standard test organisms (*3*, *7*). These in vivo studies, conducted for a relatively short period (less than 48-hr exposure), have generally estimated metabolite formation as a dose-independent first-order kinetic process yielding a metabolite formation rate constant ($k_m$, h$^{-1}$) and a metabolite elimination rate constant ($k_{em}$, h$^{-1}$). A few studies have examined the impact of biotransformation on the toxicity of PAHs to aquatic invertebrates based on measured body residues of parent compound and metabolites (*10*, *11*). Generally, the impact of biotransformation on the toxic response and bioconcentration has been examined using a known biotransformation inhibitor, piperonyl butoxide (PBO) (*12*−*17*). However, there is no study on the biotransformation inhibition for PAH in aquatic invertebrates. It is apparent that a biotransformation inhibition experiment can be a useful tool to answer the following question: What is the real cause of PAH toxicity to aquatic invertebrates, parent compound or hidden metabolites?

In the companion paper (*18*), a multicomponent damage assessment model (MDAM) was developed to analyze the time-dependent toxicity of a mixture with toxicokinetic interactions, especially biotransformation and its inhibition. This study is to apply the MDAM to metabolized PAH. Thus, the toxicokinetics and time-dependent toxicity of parent compound and metabolites were separately determined in toxicity and toxicokinetic experiments for parent compound in the presence and absence of a biotransformation inhibitor (PBO). Since the relative contribution of PBO ($TU_I(t)$) to the total toxicity of metabolized PAH was not negligible, the $TU_I(t)$ was indirectly determined from toxicity experiments for nonmetabolized DDE in the presence and absence of PBO. Further, through a series of toxicokinetic and toxicity experiments, the toxicokinetic and toxicodynamic parameters for parent compound and metabolites were separately estimated using MDAM.

## Materials and Methods

**Experimental Designs.** The toxicokinetic and toxicodynamic parameters for parent compound and metabolites of fluorene


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and pyrene were estimated in *Hyalella azteca* by a series of experiments including short-term (24−48 h uptake and 48−72 h depuration) biotransformation experiment for PAHs without PBO, long-term (10−12 d uptake) biotransformation experiment for PAHs in the presence and absence of PBO, and toxicity experiments for PAHs and DDE in the presence and absence of PBO. Chemicals, organism, exposure media, and a detailed description of the toxicity experiments are given in the Supporting Information part I.

*Short-Term Biotransformation Experiments without PBO. H. azteca* were exposed to pyrene (0.29 $\mu$mol $L^{-1}$) and fluorene (2.5 $\mu$mol $L^{-1}$) for 48 and 24 h, respectively, and moved to clean water for elimination for 72 or 48 h, respectively. In the uptake phase, animals for pyrene were sampled at 2, 4, 6, 8, 12, 24, and 48 h and for fluorene were sampled at 1, 2, 4, 6, 8, 12, 16, and 24 h. In the depuration phase test animals were sampled at 2, 4, 6, 8, 12, 24, 48, and 72 h for pyrene, and at 1, 2, 4, 6, 8, 12, 16, 24, and 48 h for fluorene. At each sample time, 10 amphipods from each beaker ($n = 3$) were taken for total body residue, and 30 amphipods were taken for biotransformation. Total body residue was measured by liquid scintillation counting (LSC) as in the previous study (*6*).

*Long-Term Biotransformation Experiments in the Presence and Absence of PBO. H. azteca* were exposed to the same concentrations of pyrene and fluorene used in the short-term experiments for 10 and 12 d in the presence and absence of PBO (0.89 $\mu$mol $L^{-1}$). Test animals were sampled at 1, 2, 4, 8, and 12 d for pyrene and at 1, 2, 4, 7, and 10 d for fluorene. At every sampling time, 10 amphipods from each beaker ($n = 3$) were analyzed for total body residue, and 30 amphipods were taken for biotransformation. Dead animals, defined as animals with no response against a stimulus by prodding with a glass tube, were also retrieved for measurement of total body residue.

*Biotransformation Measurement.* Three composite samples of twenty amphipods in each sampling time were blotted dry, weighed (±0.001 mg), and extracted with 1.5 mL of ethyl acetate/acetone (1:4, v:v) using a Ten Broeck glass tissue grinder. The residual tissue was re-extracted twice with 2 mL of cyclohexane. The extract was filtered through a glass-fiber filter (GFF 0.45 $\mu$m, Whatman) to separate the nonextracted (tissue bound) fraction. Tissue solubilizer (0.5 mL, Soluene-350, Packard Bioscience, Meridan, CT) was added to the glass-fiber filter and held overnight. Scintillation cocktail (12 mL, 3a70b, Research Products International, Mt. Prospect, IL) was added, and the radioactivity was determined by liquid scintillation counting (LSC). The organic extract was passed over anhydrous sodium sulfate, the volume was reduced under a stream of nitrogen to about 6 mL, and an aliquot of 200 $\mu$L was taken for total radioactivity. The extract was analyzed by thin-layer chromatography (TLC) on precoated silica gel 60F-254Em glass plates (Alltech Associates, Deerfield, IL) using hexane/benzene (8:2, v/v) for determination of parent compound and polar metabolites. Developed plates were divided into several segments, and the silica gel was removed and transferred to a 12-mL scintillation cocktail. After subsidence of chemiluminescence for 24 h in the dark, the [$^{14}$C] activity was determined by LSC. The fraction of metabolites was determined as the sum of the tissue-bound residue and the nonparent activity on the TLC plate and was divided by the total expected activity based on total body residue measurements by LSC (*6*). The relative fraction of parent PAHs in the exposed organisms was determined as the difference between the expected total and the measured amount of metabolites. The mass balances calculated as the sum of tissue residual and the extracted compound compared to the expected total body residue ranged from 51 to 130% (86 ± 19% (mean ± standard deviation), $n = 47$) for pyrene and from 60 to 86% (75 ± 10%, $n = 12$) for fluorene.

*Toxicity Experiments in the Presence and Absence of PBO.* Water-only toxicity experiments to PAH with and without co-exposure to PBO (0.89 $\mu$mol $L^{-1}$) used a static-renewal exposure. For fluorene (treatment levels 3.1 ± 0.1, 4.2 ± 0.1, 5.4 ± 0.2, 7.8 ± 0.2 $\mu$mol $L^{-1}$), pyrene (treatment levels 0.35 ± 0.01, 0.44 ± 0.02, 0.55 ± 0.02, 0.69 ± 0.02 $\mu$mol $L^{-1}$), and DDE with PBO (13 ± 0.4, 17 ± 1, 24 ± 1, 40 ± 1 $\mu$mol $L^{-1}$) and without PBO (21 ± 1, 25 ± 1, 28 ± 2, 57 ± 2 $\mu$mol $L^{-1}$), toxicity experiments were conducted for 10 days with three replicates per treatment.

**Data Analysis.** *Toxicokinetic Modeling for PAHs.* The toxicokinetics of an organic chemical accumulated from water with biotransformation can be described by coupled equations with first-order elimination of parent compound and metabolites and Michaelis−Menten biotransformation (*18*). Since PAH such as fluoranthene, pyrene, and fluorene in *Hyalella azteca* did not show any dose-dependent toxicokinetic parameters (*6*, *19*), a simple first-order toxicokinetic model was used to describe the biotransformation process. If the body residue of parent compound is much smaller than the half-saturation concentration ($K_M$) (this situation is similar to the exposure to low water concentration of PAH), the biotransformation toxicokinetics become similar to first-order kinetics as follows:

$$\frac{dC_p}{dt} = k_u C_w - (k_{ep} + k_m) C_p \quad (1)$$

$$\frac{dC_m}{dt} = k_m C_p - k_{em} C_m \quad (2)$$

where $C_w$, $C_p$, and $C_m$ are the concentrations in the exposure water ($\mu$mol $L^{-1}$), the organism concentrations of parent compound ($\mu$mol $kg^{-1}$), and the metabolites ($\mu$mol $kg^{-1}$), respectively. The $k_u$ is the uptake rate coefficient (L $kg^{-1}$ $h^{-1}$). Elimination processes for the parent compound include first-order kinetics ($k_{ep}$) for diffusion loss and biotransformation ($k_m$). For simplicity, the elimination process for the metabolites was described as a first-order loss process ($k_{em}$, $h^{-1}$).

From the short-term biotransformation experiment, $k_u$, $k_{ep}$, $k_{em}$, and $k_m$ were estimated for pyrene using eq 1. However, for fluorene, most of the body residue data measured during the uptake phase had already reached steady state. Thus, $k_{em}$ and $k_m$ were estimated from the 10-day biotransformation experiment using the following equation:

$$\frac{dC_m}{dt} = k_m C_{p,ss} - k_{em} C_m \quad (3)$$

where the average value of body residue of parent compound for multiple exposure times was used as the steady-state body residue ($C_{p,ss}$). The $k_u$ and $k_{ep}$ for fluorene were estimated from the depuration phase in short-term biotransformation experiment (see Supporting Information part II for details).

In this study, only total body residues were measured for all treatment levels, whereas the percent metabolites and toxicokinetic parameters ($k_u$, $k_{ep}$, $k_{em}$, $k_m$) were determined only from a single treatment concentration in the short- and long-term biotransformation experiments. Therefore, further tests were required to determine whether the toxicokinetics of pyrene and fluorene were dose-dependent. Body residues for the parent compound ($C_p$) and the $BCF_p$ were calculated (see below) to examine the dose-dependence of the biotransformation toxicokinetics.

Since most of the body residue of parent compound was at steady state, the dose-dependence of the toxicokinetics for PAH was checked by calculating $BCF_p$ for several exposure concentrations ($C_w$). The $BCF_p$ was calculated without

assumption of dose-independent biotransformation, and assuming constant uptake and elimination rates as

$$\mathrm{BCF}_p = \frac{1}{(k_{\mathrm{ep}} - k_{\mathrm{em}})}\left(k_{\mathrm{u}} - \frac{C_{\mathrm{total}}(t)}{C_{\mathrm{w}}}\frac{k_{\mathrm{em}}}{(1 - e^{-k_{\mathrm{em}}t})}\right) \quad (4)$$

where $k_{\mathrm{u}}$, $k_{\mathrm{ep}}$, and $k_{\mathrm{em}}$ were estimated from the short-term biotransformation experiment (Supporting Information part III). If there is no significant correlation between $\mathrm{BCF}_{\mathrm{p}}$ and $C_{\mathrm{w}}$, the toxicokinetics are dose-independent, and the MM-type kinetic model (*18*) can be reduced to the first-order kinetic form (eq 1). However, $\mathrm{BCF}_{\mathrm{total}}$ cannot be used to check the dose-dependence of the biotransformation, because $\mathrm{BCF}_{\mathrm{total}}$ must be constant if $k_{\mathrm{em}}$ equals $k_{\mathrm{ep}}$ (eq 13 in *18*) regardless of the dose-dependence of the toxicokinetics.

*Determination of the Median Lethal Body Residue (LBR$_{50}$(t)).* The $\mathrm{LBR}_{50}(t)$ is defined as the median lethal body residue for 50% mortality at exposure time $t$ when the water concentration is constant. To assess time-dependent toxicity based on body residue, lethal body residues were measured recording time-to-death. This data set (pairs of lethal body residue and time-to-death) show variation within and between treatments; the lethal body residue increases within a treatment before the body residue reaches steady state (*6*). However, the average lethal body residue increased with decreasing median lethal time for each treatment. The median value of lethal body residue ($\mathrm{LBR}_{50}(t)$) in each treatment was matched to the median lethal time and then compared among treatments. When $t$ is the median lethal time ($\mathrm{LT}_{50}$) in treatment $i$ with water concentration $C_{\mathrm{w,i}}$, $\mathrm{LC}_{50}$-($t = \mathrm{LT}_{50,\mathrm{i}}$) is equal to $C_{\mathrm{w,i}}(\mathrm{LT}_{50,\mathrm{i}})$. So $\mathrm{LBR}_{50}(t)$ is given by

$$\mathrm{LBR}_{50}(t = \mathrm{LT}_{50,\mathrm{i}}) = R_{\mathrm{i}}(\mathrm{LT}_{50,\mathrm{i}}(C_{\mathrm{w,i}})) \quad (5)$$

where $R_{\mathrm{i}}(\mathrm{LT}_{50,\mathrm{i}}(C_{\mathrm{w,i}}))$ is the body residue when $t$ is the median lethal time ($\mathrm{LT}_{50}$) in treatment $i$ with water concentration $C_{\mathrm{w,i}}$.

*Estimation of Toxicodynamic Parameters for Parent Compound and Metabolites.* According to the MDAM (see Supporting Information part IV, *18* and *20*), the median lethal body residue ($\mathrm{LBR}_{50,\mathrm{p0}}(t)$) and the median lethal concentration ($\mathrm{LC}_{50,\mathrm{p0}}(t)$) in the absence of biotransformation can be calculated as

$$\mathrm{LBR}_{50,\mathrm{p0}}(t) = \mathrm{LBR}_{50,\mathrm{p|I}}(t)/(1 - \mathrm{TU}_{\mathrm{I}}(t)) \quad (6)$$

$$\mathrm{LC}_{50,\mathrm{p0}}(t) = \mathrm{LC}_{50,\mathrm{p|I}}(t)/(1 - \mathrm{TU}_{\mathrm{I}}(t)) \quad (7)$$

$$\mathrm{TU}_{\mathrm{I}}(t) = 1 - \mathrm{TU}_{\mathrm{DDE|I}}(t) \quad (8)$$

with $\mathrm{TU}_{\mathrm{DDE|I}}(t) \equiv \mathrm{LBR}_{50,\mathrm{DDE|I}}(t)/\mathrm{LBR}_{50,\mathrm{DDE}}(t) = \mathrm{LC}_{50,\mathrm{DDE|I}}(t)/\mathrm{LC}_{50,\mathrm{DDE}}(t)$ where $\mathrm{LBR}_{50,\mathrm{p|I}}(t)$ and $\mathrm{LC}_{50,\mathrm{p|I}}(t)$ are the median lethal body residue and the median lethal concentration in the presence of PBO, $\mathrm{LBR}_{50,\mathrm{DDE|I}}(t)$ and $\mathrm{LBR}_{50,\mathrm{DDE}}(t)$ are the median lethal body residue of DDE in the presence and absence of PBO, and $\mathrm{LC}_{50,\mathrm{DDE|I}}(t)$ and $\mathrm{LC}_{50,\mathrm{DDE}}(t)$ are the median lethal concentration of DDE in the presence and absence of PBO.

According to the damage assessment model (DAM,*20*), the toxic unit of PBO ($\mathrm{TU}_{\mathrm{I}}(t)$) defined as $C_{\mathrm{I}}(t)/\mathrm{LBR}_{50,\mathrm{I}}(t)$, is given by

$$\mathrm{TU}_I(t) = \frac{C_{\mathrm{w,I}}\mathrm{BCF}_{\mathrm{I}}K_{\mathrm{I}}(t)}{D_{\mathrm{L}}/(k_{\mathrm{aI}}/k_{\mathrm{rI}})/P_{\mathrm{I}}(t)} = \frac{C_{\mathrm{I,ss}}}{\mathrm{LBR}_{50,\mathrm{I}}(t = \infty)}K_{\mathrm{I}}(t)P_{\mathrm{I}}(t) \quad (9)$$

where $C_{\mathrm{I}}(t) = C_{\mathrm{w,I}}\mathrm{BCF}_{\mathrm{I}}K_{\mathrm{I}}(t)$, $\mathrm{LBR}_{50,\mathrm{I}}(t) = D_{\mathrm{L}}/(k_{\mathrm{aI}}/k_{\mathrm{rI}})/P_{\mathrm{I}}(t)$, $C_{\mathrm{I,ss}} = C_{\mathrm{w,I}}\mathrm{BCF}_{\mathrm{I}}$, $\mathrm{LBR}_{50,\mathrm{I}}(t = \infty) = D_{\mathrm{L}}/(k_{\mathrm{aI}}/k_{\mathrm{rI}})$, $K_{\mathrm{I}}(t) = 1 - e^{-k_{\mathrm{eI}}t}$, $P_{\mathrm{I}}(t) = k_{\mathrm{rI}}((e^{-k_{\mathrm{eI}}t} - e^{-k_{\mathrm{rI}}t})/(k_{\mathrm{eI}} - k_{\mathrm{rI}}) + (1 - e^{-k_{\mathrm{rI}}t})/k_{\mathrm{rI}})/(1 - e^{-k_{\mathrm{eI}}t})$, $k_{\mathrm{aI}}$ and $k_{\mathrm{rI}}$ are the damage accrual rate coefficient and the damage repair rate constant for the inhibitor, respectively, and $D_{\mathrm{L}}$ is the damage level for 50% mortality,which equals ln 2.

If the toxicokinetic parameters are determined, toxicodynamic parameters for parent compound can be estimated using eqs 10 and 11 for $\mathrm{LBR}_{50,\mathrm{p0}}(t)$ and $\mathrm{LC}_{50,\mathrm{p0}}(t)$ as follows:

$$\mathrm{LBR}_{50,\mathrm{p0}}(t) = \frac{D_{\mathrm{L}}/k_{\mathrm{ap}}}{\frac{1}{(1 - e^{-k_{ept}})}\left(\frac{e^{-k_{ept}} - e^{-k_{rpt}}}{k_{\mathrm{ep}} - k_{\mathrm{rp}}} + \frac{1 - e^{-k_{rpt}}}{k_{\mathrm{rp}}}\right)} \quad (10)$$

$$\mathrm{LC}_{50,\mathrm{p0}}(t) = \frac{D_{\mathrm{L}}/k_{\mathrm{ap}}}{\frac{k_{\mathrm{u}}}{k_{\mathrm{ep}}}\left(\frac{e^{-k_{ept}} - e^{-k_{rpt}}}{k_{\mathrm{ep}} - k_{\mathrm{rp}}} + \frac{1 - e^{-k_{rpt}}}{k_{\mathrm{rp}}}\right)} \quad (11)$$

where $k_{\mathrm{ap}}$ and $k_{\mathrm{rp}}$ are the damage accrual rate coefficient and the damage repair rate constant for the parent compound, respectively.

According to MDAM (*18*), toxicodynamic parameters for PAH including the damage accrual rate coefficient ($k_{\mathrm{am}}$) and the damage recovery rate constant ($k_{\mathrm{rm}}$) for metabolites can be estimated as

$$\frac{D_{\mathrm{tox}}/(k_{\mathrm{am}}/k_{\mathrm{rm}})}{P_{\mathrm{m}}{}^{1}(t)} = \frac{f_{\mathrm{m}}{}^{1}(t)}{\frac{1}{\mathrm{LBR}_{50,\mathrm{total}}(t)} - p^{1}(t)\frac{(1 - f_{\mathrm{m}}{}^{1}(t))}{\mathrm{LBR}_{50,\mathrm{p0}}(t)}} \quad (12)$$

where, if the body residue of parent PAH is steady state

$$P_{\mathrm{m}}^{1}(t) \cong \frac{k_{\mathrm{rm}}}{1 - e^{-k_{emt}}}\left(\frac{e^{-k_{emt}} - e^{-k_{rmt}}}{k_{em} - k_{rm}} + \frac{1 - e^{-k_{rmt}}}{k_{rm}}\right),$$

$$f_{\mathrm{m}}^{1}(t) \cong \frac{1 - e^{-k_{emt}}}{1 - e^{-k_{emt}} + (k_{\mathrm{em}}/k_{\mathrm{m}})}, \; p^{1}(t) \cong 1, \text{ and}$$

$$LBR_{50,p0}(t) \cong \frac{D_{tox}/(k_{ap}/k_{rp})}{1 - e^{-k_{\mathrm{rp}}t}}$$

(see Supporting Information IV for details). In the above equation, the median lethal body residues for parent and metabolites ($\mathrm{LBR}_{50,\mathrm{p}}(t)$ and $\mathrm{LBR}_{50,\mathrm{m}}(t)$) were measured from toxicity experiments in the absence of PBO, $\mathrm{LBR}_{50,\mathrm{p0}}(t)$ was calculated from $\mathrm{LBR}_{50,\mathrm{p|I}}(t)$ using eq 6, and toxicokinetic parameters for parent and metabolites were estimated from short- and long-term biotransformation experiments in the presence and absence of PBO.

The data were fit by an iterative least-squares fit to eqs 10 and 12 using the fourth-order Runga−Kutta approach with a time step 0.01 using Scientist, v. 2.01 (MicroMath, Salt Lake City, UT).

## Results and Discussion

**Toxicokinetics and Biotransformation of Pyrene and Fluorene in the Presence and Absence of PBO.** *Pyrene.* Toxicokinetic parameters for pyrene were estimated from the measured body residues for parent compound and metabolites in the short term study at $0.27 \pm 0.02$ $\mu$mol $\mathrm{L}^{-1}$ (Figure S-1 in the Supporting Information). The uptake rate coefficient ($k_{\mathrm{u}}$) ($99 \pm 9$ L $\mathrm{kg}^{-1}$ $\mathrm{h}^{-1}$), the elimination rate constant for parent compound ($k_{\mathrm{ep}}$) ($0.050 \pm 0.008$ $\mathrm{h}^{-1}$), the biotransformation rate constant ($k_{\mathrm{m}}$) ($0.016 \pm 0.003$ $\mathrm{h}^{-1}$), and the elimination rate constant for metabolites ($k_{\mathrm{em}}$) ($0.021 \pm 0.007$ $\mathrm{h}^{-1}$) were estimated (Table S-1). The $\mathrm{BCF}_{\mathrm{p}}$ and $\mathrm{BCF}_{\mathrm{total}}$ were estimated to be 1500 and 2600 L $\mathrm{kg}^{-1}$, respectively. The percent of metabolites at steady state was estimated to be 43% by $k_{\mathrm{m}}/(k_{\mathrm{m}} + k_{\mathrm{em}})$.

For the 12-d uptake phase experiment, the total body residue in live animals increased continuously (Figure 1a). There was no difference between total body residues in the

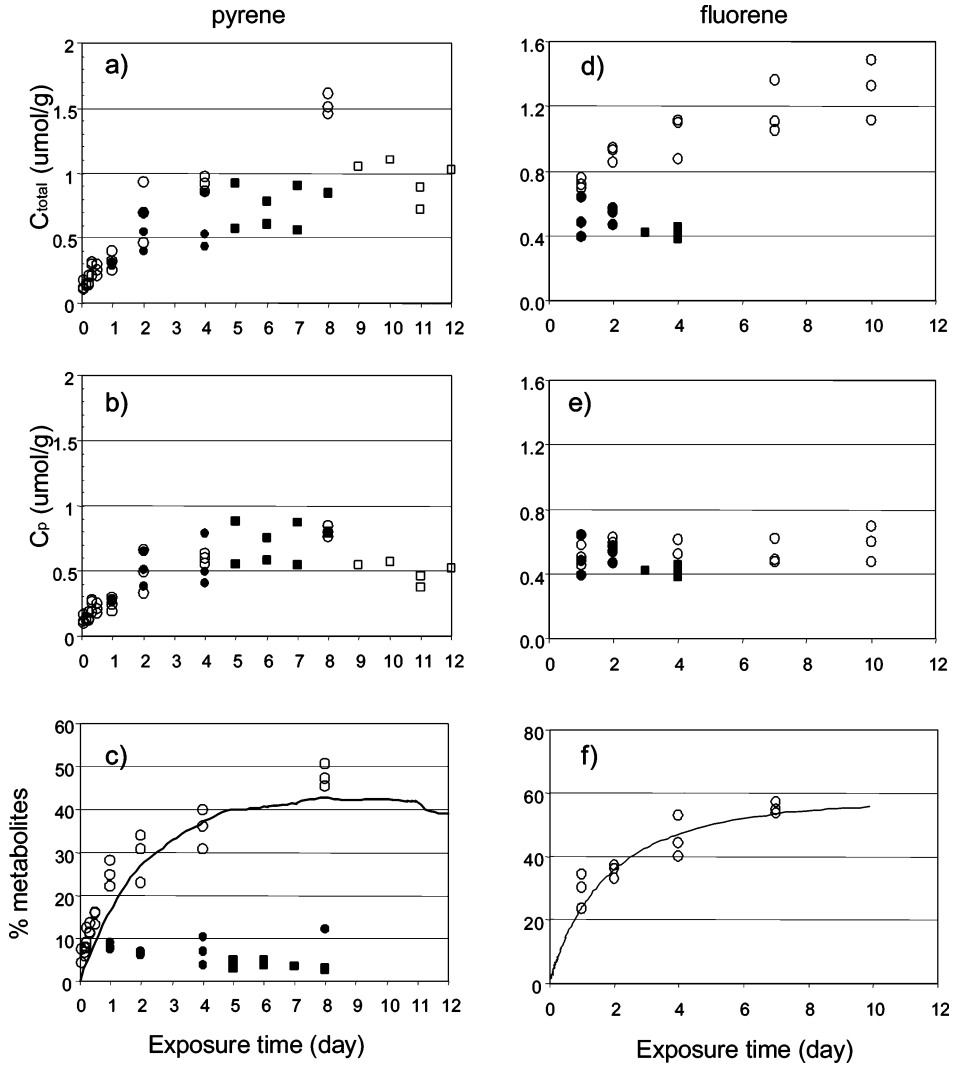


**FIGURE 1. Bioaccumulation of pyrene (0.26 ± 0.02 $\mu$mol $L^{-1}$) and fluorene (2.78 ± 0.07 $\mu$mol $L^{-1}$) in *Hyalella azteca* for 12 d in the presence and absence of piperonyl butoxide (PBO) (0.89 $\mu$mol $L^{-1}$). $C_{total}$, total body residue; $C_p$, body residue of parent compound. Open marks (○ and □) are for data in the absence of PBO and closed marks (● and ■) are for data in the presence of PBO. Circles (○ and ●) are for live animals, and rectangles (□ and ■) are for dead animals. The fitted curve for the percent of metabolites of pyrene was calculated using eq S-7 in Supporting Information part IV. The fitted curve for the percent of metabolites of fluorene is calculated using eq S-7 assuming the constant body residue of parent fluorene.**

presence and absence of PBO until day 4, but the percent of metabolites in the absence of PBO (up to 35%) was greater than that in the presence of PBO (below 10%) (Figure 1c). Total body residues at day 8 in live animals in the absence of PBO were greater than those in the presence of PBO. However, there were no differences in body residues of parent compound for the two exposure conditions (Figure 1b). Measured percent of metabolites was well fit as a function of time using the estimated $k_m$ and $k_{em}$ from the short-term biotransformation study (Figure 1c). In the presence of PBO, the concentrations of total residue and parent compound reached steady state after 5 days, when $BCF_p$ and $BCF_{total}$ were 2800 and 2900 L $kg^{-1}$, respectively. The percent of metabolites in the presence of PBO did not change from day 1 to day 8 (5.7 ± 2.7%) (Figure 1c).

*Fluorene.* For the 10-d uptake experiment, the total body residue in the absence of PBO continuously increased (Figure 1d), but total body residue in the presence of PBO reached steady state after day 1. The total body residues in the absence of PBO were greater than those in the presence of PBO, but body residues for parent compound were similar between the two exposures and reached steady state within 1 day (Figure 1e). The percent of metabolites in the absence of PBO increased to 55% by day 7, meanwhile, in the presence of PBO the percent of metabolites was below the detection limit (Figure 1f). Since the body residue for parent compound was steady state (0.55 $\mu$mol $g^{-1}$) (Figure 1e), a biotransformation rate constant ($k_m$) (0.015 ± 0.002 $h^{-1}$) and an elimination rate constant for metabolites ($k_{em}$) (0.011 ± 0.002 $h^{-1}$) for fluorene could be estimated using eq 3 (Figure 1f). The percent of metabolites in total body residue at steady state was estimated to be 58% by $k_m/(k_m + k_{em})$.

In contrast to pyrene, it was impossible to estimate the toxicokinetic parameters from the short-term biotransformation experiment, because total body residues in the 24-h uptake phase did not change ($p = 0.37$). However, the fluorene elimination rate constant could be estimated from the initial slope in the depuration phase (1.7 ± 0.2 $h^{-1}$) (see Figure S-2 in Supporting Information II). Meanwhile, a second slope (0.013 ± 0.013 $h^{-1}$), which is similar to the $k_{em}$ value (0.011 ± 0.002 $h^{-1}$), was also observed. Therefore, the rate constant calculated from the first slope was considered to be similar to the elimination rate constant for parent fluorene ($k_{ep}$). The apparent $BCF_{total}$ calculated by $C_{total}/C_w$ was 190 ± 20 L $kg^{-1}$ and did not change over the 24-h uptake ($p = 0.42$). An uptake clearance rate coefficient ($k_u$ = 130 ± 30 L $kg^{-1}$ $h^{-1}$)

was estimated using eq 4. The $BCF_p$ was calculated to be 79 ± 28 L $kg^{-1}$, which is about half of the $BCF_{total}$ and reflects the high biotransformation and the slow elimination rate of the fluorene metabolites.

*Influence of PBO on PAH Toxicokinetics.* Since most measured $C_p$ values were at steady state, apparent BCF values for fluorene ($C_p/C_w$) equaled $BCF_p$. The $BCF_p$ for pyrene in the absence of PBO (1300−1700 L $kg^{-1}$) was smaller than that in the presence of PBO (2000 ± 160 L $kg^{-1}$). Meanwhile the apparent $BCF_{total}$ ($C_{total}/C_w$) in the absence of PBO (2300 ± 180 L $kg^{-1}$) was greater than that in the presence of PBO (2000 ± 160 L $kg^{-1}$) (*t*-test, $p = 0.016$). For fluorene, the apparent $BCF_{total}$ in the presence of PBO (140 ± 16 L $kg^{-1}$) was greater than that in the absence of PBO (110 ± 16 L $kg^{-1}$) (*t*-test, $p = 0.018$). The $BCF_p$ in the presence of PBO was not different from the apparent $BCF_{total}$ in the presence of PBO, but was greater than the $BCF_p$ in the absence of PBO (78 ± 1 L $kg^{-1}$).

*Checking the Dose-Dependence of Toxicokinetics.* The dose-dependence of the toxicokinetics was not determined from the biotransformation experiments and the percent of metabolites was measured at a single treatment level because of time and resource limitations. So the dose-dependence of toxicokinetics needed to be evaluated to determine whether the measured percent of metabolites could be applied to other exposure concentrations. For both pyrene and fluorene, neither $BCF_{total}$ nor $BCF_p$ values calculated from eq 4 showed any correlation with $C_w$. Fluorene toxicokinetics were assumed to be dose-independent, because the relative contribution of biotransformation in total fluorene elimination (less than 1%, based on the calculation by $k_m/(k_{ep}+k_m)$ and assuming that $k_{ep}$ is 1.7 $h^{-1}$) was estimated to be 1 order of magnitude smaller than pyrene (24%). Thus, it is reasonable that the toxicokinetics for PAH biotransformation in *H. azteca* are dose-independent. Therefore, the percent of metabolites measured in a single dose was applied to the $LBR_{50}(t)$ measured at several treatment levels in the toxicity experiments.

The toxicokinetic parameters for pyrene and fluorene, estimated here, were different from those estimated using a one-compartment first-order kinetic model without biotransformation in the previous study (*6*) (Table S-1). Results for pyrene in this study were similar to the toxicokinetic parameters for fluoranthene in *H. azteca* (*7*), whose octanol−water partition coefficient (log $K_{OW} = 5.22$; *21*) is similar to that for pyrene (log $K_{OW} = 5.18$; *21*). In the case of fluorene, even though the biotransformation rate constant was similar to pyrene or three times smaller than that for fluoranthene, the relative contribution of biotransformation in total elimination process of fluorene (less than 1%) was much smaller than for either pyrene (24%) or fluoranthene (55%), because of the estimated very fast elimination of parent fluorene.

**Time-Dependence of Toxicity of Pyrene and Fluorene in the Presence and Absence of PBO.** Mortalities by pyrene and fluorene in the presence of PBO were greater than those in the absence of PBO. The differences in mortality tended to be magnified with decreasing PAH water concentration (Figure 2). Even though there was no mortality at 3.1 μmol $L^{-1}$ of fluorene and 0.35 μmol $L^{-1}$ of pyrene in the absence of PBO by day 10, most test animals in both treatments in the presence of PBO were dead at day 10.

The slopes of log $C_w$ or log $LBR_{50}$ vs log $LT_{50}$ in the presence of PBO were steeper than those in the absence of PBO (Figure 3). On the basis of the slope and magnitude of the $LBR_{50}(t)$ values for total body residue of pyrene in the presence and absence of PBO, the toxicity did not appear to be controlled by the total body residue. The $LBR_{50}(t)$ values for parent pyrene in the presence and absence of PBO showed a trend similar to those for total body residue, but the magnitudes

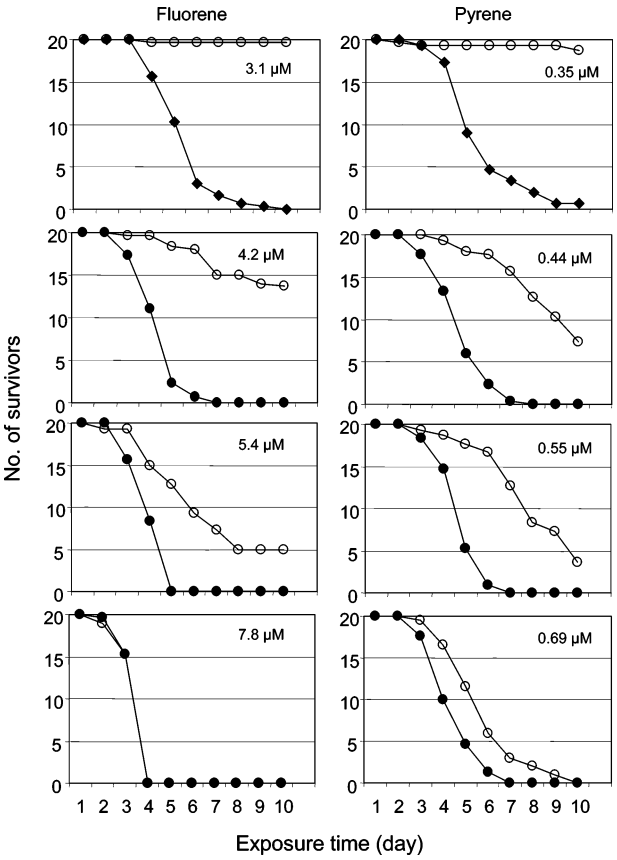


**FIGURE 2. Toxicity of pyrene and fluorene in the presence and absence of piperonyl butoxide (PBO) (0.89 μM) to *Hyalella azteca* for 10 days. There was no mortality for the control and the PBO control for 10 d. Symbols: ○, the number surviving in the absence of PBO; and ●, the number surviving in the presence of PBO. Numbers are water concentrations of pyrene and fluorene.**

of the differences were smaller (Figure 3). Similar to pyrene, $LBR_{50}(t)$s for total body residue of fluorene in the presence and absence of PBO also showed a trend similar to those for pyrene, but $LBR_{50,p|I}(t)$ for parent fluorene in the presence of PBO was greater than $LBR_{50,p}(t)$ in the absence of PBO (Figure 3).

The median lethal body residue producing 50% mortality ($LBR_{50}(t)$) was determined in the presence and absence of PBO as the lethal body residue ($C_p$) at $t = LT_{50}$ in each treatment. For pyrene and fluorene, their body residues reached steady state within 24 h, therefore, median values of lethal body residues (mean lethal residue, $MLR_{50}(t = LT_{50})$) in each treatment equaled the $LBR_{50}(t)$. However, for DDE, the $MLR_{50}(t)$ was greater than the $LBR_{50}(t)$, because lethal body residues did not reach steady state over the course of the exposure (Figure 3). The measured body residues for DDE in dead animals increased with increasing individual time-to-death within a treatment, whereas the lethal body residue decreased with increasing time-to-death among treatments at a fixed mortality level (see ref *20* for details).

Both the $LBR_{50}(t)$s and $LC_{50}(t)$ for DDE in the absence of PBO were greater than in the presence of PBO (Figure 3). The lower the water concentration ($C_w$), the greater the difference between $LBR_{50}(t)$s or $LC_{50}(t)$s in the presence and absence of PBO. The toxic unit of PBO ($TU_I(t)$), representing of toxicity contribution of 0.89 μmol $L^{-1}$ PBO, increased with increasing exposure time (Figure S-3), which is due to both the decrease of the $LBR_{50}(t)$ for PBO and the increase of body residue of PBO.

The $LBR_{50,p0}(t)$, representing the intrinsic toxicity potency of parent compound, was calculated from $LBR_{50,p|I}(t)$,

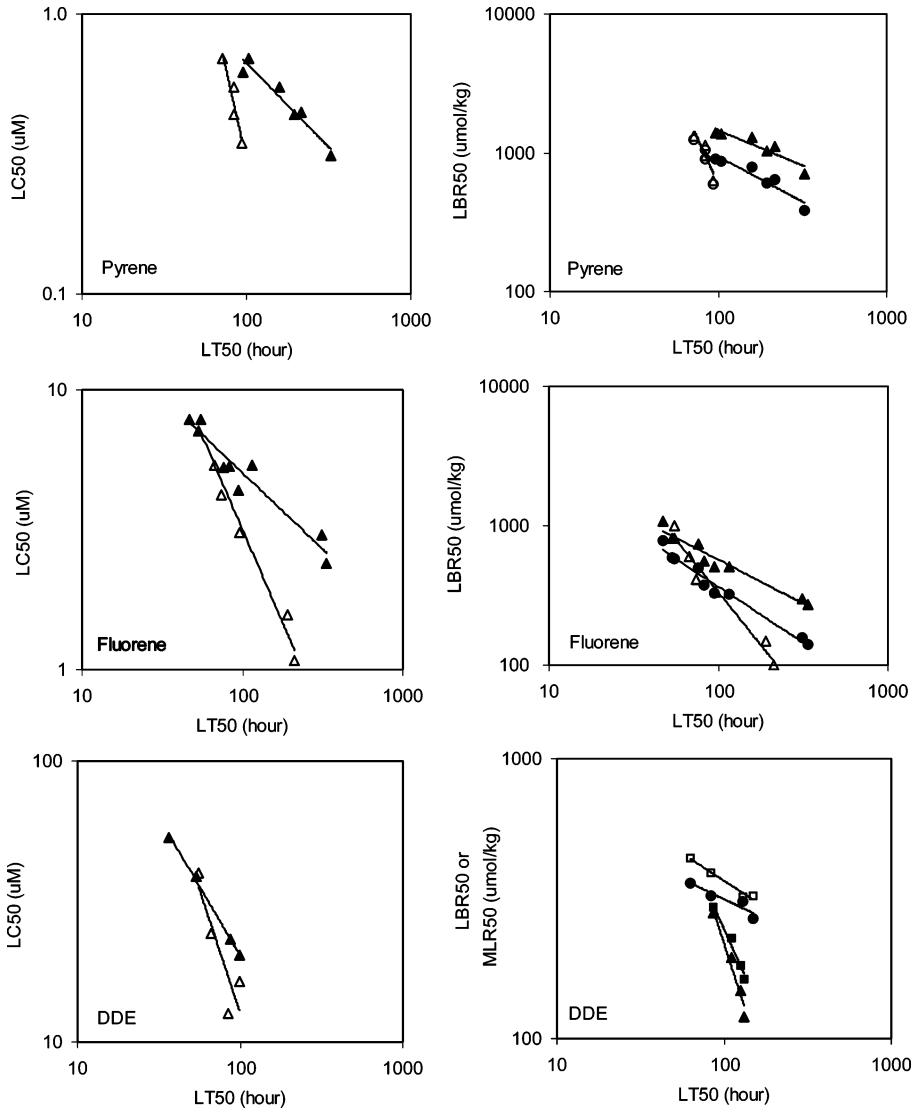


**FIGURE 3. Median lethal concentrations for 50% mortality ($LC_{50}(t)$), and median body residues for 50% mortality ($LBR_{50}(t)$) of pyrene, fluorene, and DDE in *Hyalella azteca* with and without co-exposure of piperonyl butoxide (PBO) (0.89 $\mu$mol $L^{-1}$). The symbols ◇ and ◆ are $LC_{50}(t)$ in the presence and absence of PBO. The symbols ○ and ● are $LBR_{50}(t)$ for body residue of parent compound in the presence and absence of PBO, and △ and ▲ are $LBR_{50}(t)$ for total body residue in the presence and absence of PBO. The symbols □ and ■ are the mean value of lethal body residue ($MLR_{50}(t)$) matched with the median lethal time ($LT_{50}$) for DDE in the presence and absence of PBO.**

$LBR_{50,DDE|I}(t)$, and $LBR_{50,DDE}(t)$ using eq 6. For pyrene, the $LBR_{50,p0}(t)$ was similar to the $LBR_{50,p}(t)$ representing mixture toxicity of parent pyrene and metabolites (Figure 4a). Thus, it indicates that the toxicity of the metabolites is negligible ($TU_m(t) \cong 0$). In contrast, the $LBR_{50,p}(t)$ for fluorene was greater than $LBR_{50,p0}(t)$ (Figure 4b), supporting the hypothesis that the toxicity of metabolites is significant ($TU_m(t) > 0$).

Mixture toxicity of PAH and PBO looks like a synergistic effect with PBO enhancing the toxicity of PAH (Figure 2). This is due to an increase in BCF of parent PAH in the presence of PBO. However, if the results in Figure 2 were analyzed by MDAM based on body residue, the mixture toxicity of PAH and PBO is not synergistic, but appears to be additive. Note that the difference of the velocity in which the body residues reach steady state in the presence and absence of PBO can make the mixture toxicity deviate from a simple additive effect (*18*), based on the difference of the velocity increase with an increasing ratio of $k_m/(k_{ep} + k_m)$.

**Estimation of Toxicodynamic Parameters for Parent Compound and Metabolites.** Theoretically, to estimate the damage accrual rate coefficient ($k_{ap}$) and the damage repair rate constant ($k_{rp}$) for the parent compound, we must know $LBR_{50,p0}(t)$ and $LC_{50,p0}(t)$, which are the median lethal residue and median lethal concentration assuming the absence of biotransformation and without inhibitor present. For pyrene, the time window of the $LBR_{50,p0}$ and $LBR_{50,p}$ unfortunately did not overlap. So it is impossible to directly compare $LBR_{50,p0}$ and $LBR_{50,p}$. However, the minimum values of $LBR_{50,p0}(t)$ could be determined assuming that $LBR_{50,p}(t)$ equals $LBR_{50,p0}(t)$. Note that $LBR_{50,p0}(t)$ cannot be smaller than $LBR_{50,p}(t)$, because the intrinsic toxicity of parent compound does not change.

The $k_{ap}$ and $k_{rp}$ for parent pyrene that were estimated from the $LBR_{50,p0}(t)$ assuming that $LBR_{50,p}(t)$ equals $LBR_{50,p0}(t)$ were comparable with those estimated from only the $LBR_{50,p}(t)$ (Table 1). The $LBR_{50,p0}(t)$ and $LBR_{50,p}(t)$ overlapped (Figure 4a). There is no significant difference in toxicodynamic parameters in the presence and absence of PBO. So it is reasonable to assume that the $LBR_{50,p}(t)$ for pyrene equals the $LBR_{50,p0}(t)$. For fluorene, since $LBR_{50,p0}(t)$ is greater than $LBR_{50,p}(t)$, the $k_{ap}$ and $k_{rp}$ for parent fluorene were estimated only from the $LBR_{50,p0}(t)$ calculated from $LBR_{50,p|I}(t)$ (Table 1).

The $LBR_{50,p0}$ for parent pyrene at $t = \infty$ ($LBR_{50,p}(t = \infty) = 0.24 \pm 0.24$ $\mu$mol $g^{-1}$) given by $(D_L/k_{ap})k_{rp}$ was about three

**TABLE 1. Toxicodynamic Parameters Based on Body Residue of Parent Compound and Total Body Residue of Narcotic Compounds in Aquatic Invertebrates**

| compound | species | $k_a$ or $k_{ap}$ ($\mu$mol$^{-1}$ g h$^{-1}$) (avg ± std) | $k_r$ or $k_{rp}$ (h$^{-1}$) (avg ± std) | $LBR_{50}(t=\infty)$ ($\mu$mol g$^{-1}$) (avg ± std)[g] |
|---|---|---|---|---|
| | | **parent compound** | | |
| pyrene[a] | *H. azteca* | 0.009 ± 0.001 | 0.003 ± 0.003 | 0.24 ± 0.24 |
| pyrene[a] | *H. azteca* | 0.012 ± 0.002 | 0.008 ± 0.002 | 0.45 ± 0.12 |
| fluorene[a] | *H. azteca* | 0.020 ± 0.006 | 0.013 ± 0.008 | 0.46 ± 0.31 |
| DDE[b] | *H. azteca* | 0.062 ± 0.014 | 0.037 ± 0.014 | 0.41 ± 0.19 |
| PCBz[c] | *H. azteca* | 0.038 ± 0.006 | 0.021 ± 0.006 | 0.38 ± 0.13 |
| DBS[d] | *C. riparius* | 0.014 ± 0.002 | 0.003 ± 0.002 | 0.15 ± 0.11 |
| | | **metabolite** | | |
| fluorene | *H. azteca* | 0.103 ± 0.034 | 0.034 ± 0.021 | 0.33 ± 0.23 |
| | | **total body residues** | | |
| fluorene[e] | *H. azteca* | 0.017 ± 0.001 | 0.006 ± 0.002 | 0.25 ± 0.08 |
| phenanthrene[e] | *H. azteca* | 0.025 ± 0.005 | 0.012 ± 0.005 | 0.32 ± 0.10 |
| pyrene[e] | *H. azteca* | 0.008 ± 0.001 | 0.010 ± 0.003 | 0.88 ± 0.31 |
| fluoranthene[f] | *H. azteca* | 0.003 ± 0.0003 | 0.004 ± 0.0003 | 0.84 ± 0.10 |
| fluoranthene[f] | *C. tentans* | 0.051 ± 0.010 | 0.016 ± 0.010 | 0.22 ± 0.15 |
| fluoranthene[f] | *Diporeia* spp. | 0.001 ± 0.0001 | 0.003 ± 0.0001 | 3.16 ± 0.60 |

[a] This study, these values were estimated both in the presence and absence of PBO (first row), and in the absence of PBO (second row). [b] Landrum et al. (*22*). [c] Landrum et al. (*23*). [d] Hwang et al. (*24*). [e] Lee et al. (*6*). [f] Schuler et al. (*11*). [g] Avg, averaged value; std, standard deviation; std values were calculated by propagation of errors, $std(AB) = (A^2std(B)^2+B^2std(A)^2)^{1/2}$; DDE, dichlorophenylchloroethylene; PCBz, pentachlorobenzene; DBS, dodecylbenzene sulfonate; $k_a$ or $k_{ap}$, the damage accrual rate coefficient for total body residue and parent compound, respectively; $k_r$ or $k_{rp}$, the damage recovery rate constant for total body residue and parent compound, respectively; $LBR_{50}(t = \infty)$, the median lethal residue for 50% mortality at the infinite time.

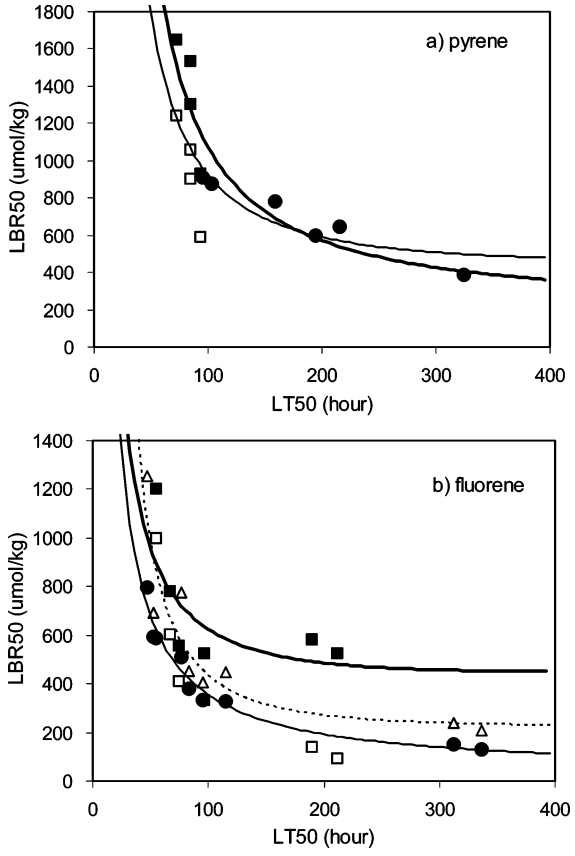


**FIGURE 4. Median lethal body residues for the toxicity of pyrene (a) and fluorene (b) in the presence ($LBR_{50,p}(t)$, □) and absence ($LBR_{50,p|I}(t)$, ●) of piperonyl butoxide (PBO) and in the absence of biotransformation ($LBR_{50,p0}(t)$, ■) in *Hyalella azteca*. The thick line is the fitting result for $LBR_{50,p0}(t)$, and the thin line is the fitting result for $LBR_{50,p}(t)$ by eq 10. Therefore, the thin line represents $LBR_{50,p0}(t)$ when the toxicity of metabolites is negligible. The dotted line is the fitting result for the median lethal body residue of fluorene metabolites (△) by eq 12.**

times smaller than $LBR_{50}$ for total body residue at $t = \infty$ ($LBR_{50}(t=\infty) = 0.88 \pm 0.31$ $\mu$mol g$^{-1}$) given by $(D_L/k_a)k_r$ (*20*). The estimated $LBR_{50,p0}(t = \infty)$ value for parent pyrene was similar to those of DDE (0.41 ± 0.19 $\mu$mol g$^{-1}$; *22*) and PCBz (0.38 ± 0.13 $\mu$mol g$^{-1}$; *23*) in *H.* azteca, whereas the $k_{ap}$ and $k_{rp}$ for pyrene were smaller than those for DDE and PCBz (Table 1).

For fluorene, the $LBR_{50,p0}(t = \infty)$ for parent fluorene (0.46 ± 0.31 $\mu$mol g$^{-1}$) was two times greater than that for total body residue (0.25 ± 0.08 $\mu$mol g$^{-1}$). This value of $LBR_{50,p0}$-$(t = \infty)$ for parent fluorene was similar to those of the other narcotic compounds such as pyrene, DDE, and PCBz in *H.* azteca (*22, 23*). Meanwhile, the $LBR_{50}(t = \infty)$ for total body residue (0.25 ± 0.08 $\mu$mol g$^{-1}$) was not similar to those of pyrene (0.88 $\mu$mol g$^{-1}$) and fluoranthene (0.84 $\mu$mol g$^{-1}$) in *H. azteca,* but was similar to that of fluoranthene (0.22 $\mu$mol g$^{-1}$) in *Chironomus tentans* (*11*), where fluoranthene metabolites are believed to contribute to the toxicity. A further study will be required to better identify the individual toxic metabolites.

In Table 1, it is notable that for PAHs the $k_{rp}$ values among three test animals (*H. azteca*, *Chironomus tentans*, and *Diporeia* spp.) ranged from 0.003 to 0.016 h$^{-1}$, whereas the $k_{ap}$ values scattered more widely from 0.001 to 0.051 $\mu$mol$^{-1}$ g h$^{-1}$ (*6, 11, 22, 23*). The range of $k_{rp}$ among test animals was smaller than that of $k_{ap}$. The difference of $k_{ap}$ values among test animals is likely due to the formation of toxic metabolites (fluoranthene in *C. tentans*; *11*) or high content of lipid in test animals (fluoranthene in *Diporeia* spp;. *11*).

The toxicodynamic parameters for fluorene metabolites ($k_{am}$ and $k_{rm}$) were first estimated using eq 10 (Figure 4). However, for pyrene metabolites, since the toxicity of pyrene metabolites was negligible, it was not possible to estimate parameters. Meanwhile, the toxicity of fluorene metabolites was represented by $k_{am}$ and $k_{rm}$, where it was required to use a lumped parameter representing all fluorene metabolites because the individual metabolites of fluorene were not measured. The estimated values for $k_{am}$ and $k_{rm}$ are 0.10 ± 0.03 $\mu$mol$^{-1}$ g h$^{-1}$ and 0.034 ± 0.021 h$^{-1}$, respectively. The $LBR_{50,m0}(t = \infty)$ for fluorene metabolites was 0.33 ± 0.23 $\mu$mol g$^{-1}$. These values are similar to those for parent fluorene.

This means that fluorene metabolites include some individual metabolites which are likely more toxic than parent fluorene.

This study is the first trial to incorporate the biotransformation process in the DAM. The MDAM is a toxicokinetic−toxicodynamic model for mixture toxicity. A first-order toxicokinetic model for PAH biotransformation and first-order toxicodynamic model were applied for metabolized PAH. In this study, only two limited cases were investigated, e.g., metabolized PAH in the absence of PBO or totally blocked metabolism in the presence of PBO. Further study needs to focus on the mechanism of the biotransformation inhibition of PAH by PBO and the quantitative characteristics. If specific parameters representing the toxicokinetic interaction between PAH and PBO are estimated in future study, combined with the toxicodynamic parameters for parent compound and metabolites determined in this study, the mixture toxicity of PAH and PBO can be fully described and predicted by MDAM. In addition, further study is required to establish dose-dependent toxicokinetics, e.g., Michaelis−Menten type kinetics for biotransformation under non-steady-state body residues. Theoretically, other cases of toxicokinetic interactions such as competitive, noncompetitive, and uncompetitive inhibition, or biotransformation induction can be combined with the MDAM based on the damage addition hypothesis.

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## Supporting Information Available

Experimental procedures, estimation of the elimination rate constant for fluorene, determination of bioconcentration factor for parent compound, and MDAM for PAHs. This material is available free of charge via the Internet at http://pubs.acs.org.

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